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STAIN TECHNOLOGY

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STAIN TECHNOLOGY

VOLUME II OCTOBER, 1927 NUMBER 4

PROGRESS IN THE STANDARDIZATION OF STAINS

CERTAIN LESS COMMONLY USED STAINS

In the work on standardization of stains, attention was naturally first given to the dyes which were in greatest demand among biologists; and the bulk of the work undertaken has been upon these more commonly used stains. Complaints were soon received, however, about stains that were more rarely employed; and altho it was not possible to give so much attention to these at the outset, it was realized that each of them presented important problems that must be solved in the future. Each of them is ordinarily called for in some particular technic which may have great value, even tho not used in many laboratories.

Little by little, information has been collected about these less commonly used stains; and altho their standardization has not been put on any scientific basis, it is now possible in most instances to obtain reliable products. It is proposed here to discuss four or five such stains, to show what information concerning them is at hand and what has been done by the manufacturers looking toward the improvement of the supplies available.

BRILLIANT CRESYL BLUE

Brilliant cresyl blue is a dye of the oxazin series, a group of dyes closely related to the thiazins, or in other words the methylene blue group. It is not at the present time employed in textile work or in any other line of commercial dyeing and hence must be prepared specially for the biologist. Its manufacturers, altho willing to cooperate to any reasonable extent, are naturally reluctant to carry on much experimental work with this dye unless they are sure the results will justify the outlay.

The dye has been employed to some extent in vital staining; but it is best known for its use as a blood stain to bring out platelets and reticulated cells. It is not yet certain whether any sample of American manufacture has been prepared which is entirely satisfactory for the former purpose. For the latter purpose, however, certain samples submitted have proved entirely satisfactory. There have been decided variations in this respect between the different samples and it has not yet proved possible to correlate them with any differences in chemical composition. The stain cannot, therefore, be regarded as standardized in a scientific sense. It can merely be said that the batches most recently submitted to the Commission and

now on the market under the Commission certification, have been found satisfactory in blood work. There may still be room for improvement; if so, criticism from users of this stain will be greatly appreciated. It is desired to obtain as much information as possible concerning any short-comings of the present supply so as to enable the manufacturers to improve the dye in the future.

CRESYL VIOLET

Cresyl violet is a dye closely related to brilliant cresyl blue, and like the latter is not employed on a commercial scale. Its chief value in histological work is on account of its metachromatic properties. It has been employed chiefly in the examination of tumor tissue, sometimes in biopsy work where rapid results are desired and where permanent preparations are not necessary, and sometimes in the more leisurely study of fixed preparations where permanent slides are required. There is some reason to believe that a stain satisfactory for one of these two purposes may not yield good results for the other.

As stated in Biological Stains, page 52, the prewar cresyl violet studied was found to be a mixture of true cresyl violet with a dye of more reddish cast. The domestic samples now on the market are apparently true cresyl violet, without the red dye formerly in some of the foreign products. Certain domestic samples put on the market a few years ago consisted of this redder dye alone. The latter was found to be quite unsatisfactory, as it lacked the metachromatic properties of cresyl violet. The sample of true cresyl violet at present on the market seems to be entirely satisfactory for biopsy work by the technic of Williams.[1] It has, however, proved unsatisfactory in other hands in making more permanent preparations from similar material.

The situation in regard to cresyl violet remains in this unsatisfactory condition. We have assurance that the company is willing to cooperate in improving the stain, but we are not at the present time able to tell them what we wish. Here again, therefore, comments and criticisms of the present supply will be welcome from any one accustomed to employing this dye. If anyone is able to cooperate in studying the matter further, his assistance will be greatly appreciated.

BRILLIANT GREEN

Brilliant green is a dye of the diamino-triphenyl-methane series, closely related to malachite green and light green, S. F. yellowish.

[1]Williams, B. G. R. Cresylecht violet a rare dye. J. Lab. and Clin. Med. 8 No. 4. Jan. 1923. Further studies with cresylecht violet including a report of my six-second method for staining tissues. Id. 10, 312.

It is not used, to any great extent at least, as a stain, but finds frequent employment as a constituent of bacteriological media. It is used for at least three different purposes: (1) as a constituent of the brilliant green bile media for plating water in order to distinguish the colon organism from other lactose fermenting organisms; (2) it is employed in media used in searching for the typhoid organism in stools, where its value comes from its ability to inhibit the colon organism; (3) it is used as a constituent of enrichment media for the stimulation of the typhoid organism, in which it must prevent the growth of the normal colon types but not be toxic to the typhoid organism.

It has proved much more simple to find a brilliant green satisfactory for colon diagnosis in water work than for the isolation and enrichment of the typhoid organism. A recent paper by Rakieten and Rettger[2] has shown the difficulty encountered in this latter instance. From their paper, however, it will be noticed that one satisfactory American sample of brilliant green was found by them. Since the writing of the paper in question these same authors have found equally satisfactory results with another sample of brilliant green manufactured by a different company. Both of these two satisfactory samples[3] of brilliant green are now on the market under the certification numbers of CBg-3 and NBg-2.

It must be admitted that it is not known in what respect these satisfactory brilliant greens differ from those that were found unsatisfactory; hence this dye cannot be regarded as scientifically standardized. No reason can be seen, however, why users cannot obtain good results with these recent certified batches of the stain.

NEUTRAL RED

Neutral red belongs to the azin series of dyes, and stands fairly close to safranin in the matter of chemical composition. In the past, it has had considerable employment as an indicator, as it is yellow in weakly basic solutions and red in weak acids. As an indicator, it has gone somewhat out of use at present on account of the greater value of the sulphonphthaleins for this purpose. It is now employed as a counterstain in certain histological procedures, or more specially as a vital stain.

Vital staining is perhaps its most important use; and it proves that the value of any neutral red sample may be judged more critically in vital staining than in any other technic. One of the facts

[2]Rakieten, M. L. and Rettger, L. F. Brilliant green and its use in an enrichment medium in the isolation of typhoid and paratyphoid organisms. J. Infec. Dis. **41**, 93.

[3]One from the National Aniline and Chemical Co; the other from Coleman and Bell.

that came to light soon after the war was that American neutral reds were too toxic to employ in this sort of work. Recent work by Phillips and Cohen[4] has shown that the dye may be more easily purified if prepared as an iodide instead of a chloride, and the neutral red iodide prepared by these investigators seems to be a very satisfactory vital stain. Meanwhile two of the stain companies have furnished samples of the chloride which do not seem to have toxic properties when tested as vital stains.

Apparently then it is now possible to obtain satisfactory non-toxic neutral reds, altho a paper elsewhere in this issue[5] suggests that the problem is not yet wholly solved.

EOSIN, ALCOHOL SOLUBLE

Several staining procedures specify the use of eosin, alcohol soluble. The most noteworthy of these is the stain for nervous tissue used in demonstrating Negri bodies in rabid animals. Some confusion has resulted in recent years because of the uncertainty as to just what dye is indented by this designation. Ordinary yellowish eosin, frequently known as eosin water soluble, is also soluble in alcohol; and the color acid of this dye is soluble in alcohol but not in water. It proves accordingly that both of these products have been sold under the name of alcohol soluble eosin; but neither is satisfactory in the demonstration of Negri bodies.

There are two closely related compounds, the methyl and the ethyl ester respectively of yellowish eosin, either of which is more correctly known as alcohol soluble eosin. These esters are readily soluble in alcohol and in boiling water, but dissolve only very sparingly in cold water. They differ quite distinctly from the yellowish eosin, giving cherry red solutions, without appreciable fluoresence. Apparently the eosin alcohol soluble available before the war was the ethyl ester. Since this information has been obtained and passed on to the manufacturers, they have readily secured this dye for us, altho it has no commercial application at the present time.

This dye is now on the market under the name of ethyl eosin, with alcohol soluble eosin recognized as a synonym. The name ethyl eosin is preferred as it is more explicit. Two batches of this stain have been approved by the Commission and can be confidently used in the procedures for staining Negri bodies for which alcohol soluble eosin is specified.

H. J. CONN

[4]Philips, M. and Cohen, B. The preparation of neutral red iodide suitable for vital staining. Stain Tech. **2**, 17. 1927. The preparation of vital neutral red. Stain Tech. **2**, 74. 1927.

[5]Wilson, C. N. A comparative study of several samples of neutral red and their toxic effects on *Paramoecium caudatum*. Stain Techn. **2**, 115, 1927.

THE EVALUATION OF ETHYL EOSIN

W. C. Holmes and J. T. Scanlan

Contribution No. 140 from the Color and Farm Waste Division, Bureau of Chemistry and Soils, Dept. of Agric., Washington, D. C.

Abstract.—**Suitable methods for the determination of eosin and ethyl eosin in samples of the latter dye are described. They depend upon the possibility of precipitating the color acid and measuring the same gravimetrically. What proportion of the dye is actually ethyl eosin and what proportion is eosin Y can be determined by making use of the great difference in solubility of the two dyes in water.**

The following method may be employed for the determination of total dye:

Five-tenths of a gram of the sample is weighed out accurately and boiled gently for 30 minutes with 200 cc. of water containing 20cc. of normal sodium hydroxide. The solution is cooled and filtered, and the filtrate and washings are treated with 5 cc. of 6N. hydrochloric acid. The precipitate is filtered thru a weighed Gooch crucible, washed with a small amount of water containing traces of acid, and dried to constant weight at 110°C.

By this procedure ethyl eosin is converted into eosin and precipitated as the color acid of the latter dye. The color acid of eosin is almost completely insoluble in very dilute acids, and the accuracy of the method is very satisfactory.

In the absence of eosin in the sample the percentage of ethyl eosin (in the form of its anhydrous potassium salt) may be calculated by means of the following formula:[1]

$$\text{E. E.} = \frac{\text{Wt. of ppt.} \times 713.848 \times 100}{647.728 \times 0.5}$$

or

$$\text{E. E.} = \text{Wt. of ppt.} \times 220.42$$

Duplicate determinations carried out on a sample of dye submitted for certification as a biological stain gave 78.69%, 78.87%, 78.64% and 78.76%, respectively, of ethyl eosin.

[1]All formulae advanced are based upon the assumption that the analyses are carried out with samples of exactly 0.5 gram. If samples of other quantities are used it will be necessary to apply the appropriate corrections therefor.

The absence of eosin in samples of ethyl eosin should not be taken for granted. Eosin may be present as a result of incomplete ethylation or, possibly, of hydrolysis of ethyl eosin in the conversion of its color acid into its alkali salt. It may be tested for and determined by the following method:

Five-tenths of a gram of the sample is weighed out accurately, and 100 cc. of water is added. The solution is allowed to stand at least 24 hours with frequent shaking. It is then filtered thru a hardened filter and the filtrate centrifuged until clear. A 50 cc. aliquot is then diluted with an equal volume of water, 5 cc. of normal hydrochloric acid added and the precipitate treated as before.

At room temperature (26°C) 100 cc. of a saturated aqueous solution of eosin contains 44.2 grams of dye, whereas the solubility of ethyl eosin is approximately only 0.0312%. In the absence of eosin in the sample, the precipitate of the color acid of ethyl eosin obtained by the method outlined is approximately only 0.0148 grams. Any value obtained in excess of this value is indicative of the presence of eosin. The surplus weight of the precipitate over 0.0148 gram is that of color acid of eosin. Owing to the great solubility of eosin this value is representative of the entire quantity of eosin present in the sample. The percentage of eosin (as anhydrous disodium salt) may be calculated by means of the following formula:

$$\text{E.} = \frac{(\text{Wt. of ppt.} - 0.0148 \text{ gm.}) \times 691.706 \times 100 \times 2}{647.728 \times 0.5}$$

or

$$\text{E.} = (\text{Wt. of ppt.} - 0.0148 \text{ gm.}) \times 427.16$$

(see footnote 1)

This method was checked with samples of ethyl eosin containing (a) 8.37, (b) 16.74, and (c) 33.48% of eosin. The weights of precipitates obtained were (a) 0.0346 and 0.0344 grams, (b) 0.0540, (0.0527 rejected) and 0.0544 grams, and (c) 0.0938 and 0.0933 grams. The calculated percentages were (a) 8.42, (b) 16.83, and (c) 33.64% of eosin. The calculated values are approximately 0.5% in excess of the actual values. The cause of this minor discrepancy is obscure. Apparently it is advisable to multiply the calculated dye percentages by the further factor 0.995 in order to obtain more precise results.

When eosin is present it is obvious that the percentage of ethyl eosin in the sample can not be calculated directly from the weight of the precipitate obtained by the procedure outlined for the precipitation of total dye. It is necessary to make due allowance for the

eosin in the sample. If the weight of precipitate obtained by the method for total dye is denoted by P_1 and the weight of precipitate obtained by the method for the determination of eosin is denoted by P_2, the percentage of ethyl eosin in the sample may be calculated by the following formula:

$$\text{E. E.} = (P_1 - 2(P_2 - 0.0148 \text{ gm.})) \times 220.42$$

NOTE ON METACHROMASY

W. C. Holmes, *Washington, D. C.*

The hypothesis was recently advanced[1] that metachromasy is attributable to the selective absorption of dyes in the tautomeric forms which are shown by spectrophotometric analysis to exist in their aqueous solutions. An adequate explanation of metachromatic colors was afforded thereby.

It has since been learned that chloroform extracts the dye base of brilliant cresyl blue from aqueous solutions of the dye under ordinary conditions. The base is subsequently converted rapidly into the blue dye salt by the action of the carbon dioxide of the air. Under other conditions, however, the dye is absorbed from aqueous solutions by the chloroform almost entirely in the form of its violet tautomer, in which form it proves to be chemically inert.

The experimental results are of primary interest in other connections and will be discussed elsewhere, but it may be pointed out here that the phenomena have probable bearing upon metachromasy.

It is shown that chloroform may be stained either blue or violet by selective absorption of the tautomeric forms of the dye, depending upon conditions. A very close parallelism exists[2] between the behavior of chloroform and that of the protoplasm of living cells when brought in contact with aqueous solutions of dyes. In general, the cell protoplasm absorbs the dyes under the same conditions and in the same dye-forms as does chloroform. Protoplasm would be expected to absorb brilliant cresyl blue in its different tautomeric forms, accordingly, with corresponding variations in conditions. Penetration studies with living cells have afforded considerable evidence that such is the case.

The demonstration of the absorption of brilliant cresyl blue by protoplasm in the form of its blue or violet tautomer, respectively, with change in conditions, is held to lend considerable support to the hypothesis that the metachromatic staining of biological substances is attributable to the analogous absorption of tautomeric dye forms.

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[1]Holmes, W. C. The chemical nature of metachromasy. Stain Techn. **1,** 116. 1926.

[2]Irwin, Marion. Does methylene blue penetrate into living cells? Proc. Soc. Exp. Biol. and Med. **24,** 42 5–7. (1927).

THE STATUS OF WRIGHT'S STAIN AND SOME SUGGESTIONS AS TO ITS USE

L. O. Dutton

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Abstract.—**A questionnaire was sent to a number of the larger hospitals and laboratories of the United States in order to determine the degree of satisfaction experienced in employing Wright's stain. From a survey of the answers received, there seems little justification for poor results with this stain. It is probable that the great majority of unsatisfied users work in laboratories where the stain is used so seldom that proper manipulation is not always attained. The writer offers a few simple, but very important precautions to be observed.**

In view of the prevalence of complaints as to the efficiency of Wright's stain that emanate from the infrequent users, who generally buy small quantities of the prepared solution from local dealers, it has been thought advisable to determine if there is some inherent fault in solutions of the stain to account for these results. Accordingly, thru the agency of the Commission on Standardization of Biological Stains, a questionnaire was sent to a number of the larger hospitals and laboratories of the United States with a view of determining the degree of satisfaction that such large users of blood stains experienced with Wright's stain. The high degree of purity of the powdered stain that is available on the market would suggest that there is either a definite fault in some of these solutions or that the infrequent user has not sufficient material to evaluate properly the steps in the technic so as to obtain the best results. The complete questionnaire is given below:—

Hospital.....

Do you use Wright's stain for blood work?

If not, is your past experience with Wright's stain satisfactory?

What blood stain do you now use?

If you use Wright's stain, fill in the following:

1. Do you prepare your own crystals?
   a. If so, by what method? (Append description of method)
2. If you buy stain crystals, what company (manufacturer) do you patronize?
3. If you buy it in solution, from what manufacturer does it come?
4. What proportion of crystals to methyl alcohol do you use in preparing the staining solution?
5. Do you find that the stain is ready for use immediately after solution, or is an aging period necessary?
6. Is your stain stable or does it become unsatisfactory after a varying period?
7. Do you protect your staining solution from the light?

8. What time intervals are used:—
   a. With undiluted stain?
   b. With diluted stain?
   c. In washing?
9. Do you wash with distilled water, tap water, or a solution of buffer salts? In the latter case, how is the solution prepared and to what reaction is it buffered?
10. Do you find that your stain is satisfactory for malarial examinations.

A survey of the thirty-five answers received indicated that the users in this class are (ordinarily) satisfied with the results obtained. Only four of the answers indicated that the stain was not entirely satisfactory, and that it had been discarded in favor of other blood stains. Of course, it is impossible to determine from such a questionnaire just where the trouble lay in the unsatisfactory results obtained by these users. The fact that there are any poor results at all is indicative of the care that is required both in the preparation of the solution and in the staining technic.

Only four of the satisfied users of the stain manufacture their own powder. As for the remainder, the product has been obtained from various manufacturers and distributors with no difference in the results obtained.

The concentration of the stain varies from a maximum of 0.60% to a minimum of 0.16% (the usual 0.1g. to 60 cc. alcohol); the latter, used by eighteen of those sending in replies is the most commonly employed. There seems to be little difference in the staining properties of the various concentrations, as judged by the answers. It is reasonable to assume however, that the heavier concentrations must more readily produce a precipitate on the slide, that might be confusing.

Nineteen of the answers indicated that it was necessary to age the solution before use, or that better results were obtained if an aging period was allowed. The time indicated varied between one week and one month.

Twenty-seven users indicated that the staining solution was stable for a long period of time. Those solutions found to be unstable were chiefly the ones of higher concentration.

Twenty-one answers indicated the necessity or desirability of protecting the solution from the light. This factor did not seem to account for the stability or unstability of the stain, however, as only one of the unstable stains was not protected from the light.

The time of the various steps in the staining procedure varied considerably. There was no parallel between this variation and any factor covered by the questionnaire, and it is not known just what is responsible for this variation.

The character of the diluting water and the wash water also was varied. Distilled water, tap water and buffer solutions were used. This again, did not parallel any other obvious factor and it seems that it is a point which must be worked out by each laboratory for the particular stain and technic used.

Only one answer indicated that the stain was not satisfactory for malarial examinations. It is difficult for a laboratory making only an occasional malarial examination to evaluate properly a stain for this purpose. There is undoubtedly some variation in the staining properties of different strains and stages of the malarial parasites. For a stain to be satisfactory it must stain all parasites so that they are recognizable.

It seems from the survey of the answers received that there is little justification for poor results with Wright's stain. It is probable that the great majority of unsatisfied users work in laboratories where the stain is used so seldom that the proper timing of the various steps in the procedure is difficult, or some fault in technic has not been corrected.

After careful study of the various factors involved in securing a satisfactory finished Wright's stain, a few simple, but very important precautions are suggested by the writer.

1. It has been found that solutions varying between 0.5 g. and 1. g. dissolved in a pound bottle (approximately 600 cc.) of methyl alcohol are of sufficient concentration to produce good staining without too ready precipitation of the stain. It is the custom in the writer's laboratory to add the powder to the bottle of alcohol without trituration or much shaking and to allow it to age for about one month. Solutions so prepared have been found to retain their staining properties and stability for as long as one year. Small quantities are removed from this stock bottle. It seems to be desirable to retain the stock stain in the original brown bottle or to put it in a dark place. The working bottle is protected from the direct sunlight only.

2. It is, of course, essential that methyl alcohol of proper purity be employed. This should be of the highest grade, acetone free and neutral in reaction. Such alcohol is widely available on the market.

3. A factor that is often overlooked in staining blood smears is the cleanliness of the slides used. Slides that have been washed in soap or alkalies must be very carefully rinsed before drying. The same is true of slides cleaned in an acid solution. Traces of either acids or alkalies are almost sure to ruin the preparation.

4. With staining solutions that are relatively dilute (0.5 g. to 600 cc. methyl alcohol) there seems to be little rigidity

necessary as to the time allowed for the staining. In fact, it is almost impossible to overstain a blood smear. We have found that one can allow the undiluted stain to evaporate to complete dryness and by simply flooding the slide again with fresh solution, proceed to stain with fairly good results. It is necessary, however that at least 30 seconds be allowed before diluting the stain with water. After dilution, a period of three to four minutes is quite sufficient to secure a good stain. Here again, it is difficult to overstain, for, even if the diluted stain has completely evaporated, the slide can be saved by washing with a small quantity of the staining solution.

5. As to the water for diluting and washing it has been found that, in general, distilled water for dilution and tap water for washing is satisfactory. This will, of course, depend on the particular sample of distilled water and tap water. In some localities it will be necessary to use a properly buffered solution. The pH-value of such a buffer solution should generally be adjusted to about 6.6 to 6.8; this will vary, however, with the sample of stain used and must be determined by trial in each case.

The essential difference between the technic as outlined above and that commonly employed is in the greater dilution of the stain (one-half as concentrated as the weakest dilution indicated in the answers to the questionnaires). Such a concentration will not be satisfactory until aged for about one month. Its chief advantage is economy of the powder, freedom of preparations from precipitate, less intense staining of the red blood cells (which is desirable in staining for malaria) and failure to completely ruin a blood smear if, inadvertantly, the solution evaporates to dryness.

THE GERMICIDAL EFFECT OF STAINING SOLUTIONS

GLADYS A. ECKFELDT AND STEWART A. KOSER
From the Department of Bacteriology,
University of Illinois, Urbana.

ABSTRACT.—**Gentian violet, crystal violet and carbol fuchsin applied to cover slip preparations for one minute will destroy the majority of non-spore-forming bacteria and yeasts, tho they can not be relied upon to do this consistently and in all cases.**

The Gram staining procedure is more effective and non-spore-formers were never found to survive this process.

Methylene blue stains exert very little if any germicidal power and most organisms survived them readily. India ink was totally ineffective.

Several species of yeasts and yeast-like molds were killed in every instance by the Gram stain, gentian violet, crystal violet and carbol fuchsin, but survived both Loeffler's methylene blue and a plain aqueous solution of methylene blue.

The possibility of infection from stained glass slide preparations or from the wash water is of some importance, especially in those laboratories where a considerable number of pathogenic cultures are being examined daily. Tho several previous reports are available, the information upon this subject is not extensive and perhaps not generally appreciated. Papers by Jansen (1914), Rettger (1913), Thurn (1914), and Schmidt (1925) have appeared and, while there is some conflicting evidence, the general conclusion may be drawn that such types as the diphtheria bacillus, the members of the colon-typhoid group, various cocci including the pneumococcus, the chlorea vibrio and others will frequently survive the usual heat fixation and staining by fuchsin or methylene blue. The Gram stain and gentian violet stains show some degree of germicidal power and frequently only spore forms survive. The Ziehl-Neelson method which involves steaming in carbol-fuchsin apparently destroys the tubercle bacillus.

Our purpose has been to continue this work and extend it in certain directions. We have employed a series of 18 of the commoner organisms, including certain yeasts and yeast-like molds. Since the results obtained may depend somewhat upon the composition of the staining solutions and the method of application, it seems best to give our procedure in detail.

DYES AND STAINING METHODS

Some of the samples of dyes were older preparations and the actual dye content was unknown. It was impossible, therefore, to make

up all of the staining solutions from preparations which had a certain stated dye content, as recommended by the Commission on Biological Stains.[1] In so far as possible, however, their recommendations were followed. The composition of the staining solutions which were used is given in the following outline:—

Carbol-fuchsin: the well-known Ziehl's carbol-fuchsin diluted 1 to 10 with distilled water. This is commonly used in our laboratories as a simple stain in place of methylene blue or gentian violet. A certified sample of basic fuchsin was used, tho the dye content was not specified.

Loeffler's methylene blue: this was made up according to the original statement of the formula using 30 cc. of a concentrated solution of methylene blue in 95% ethyl alcohol and 100 cc. of a 1 to 10,000 solution of potassium hydroxide in distilled water.

Methylene blue: an aqueous solution prepared by adding 100 cc. distilled water to 1.5 grams of dye. This was allowed to stand two days with occasional shaking and was then filtered.

Formalin gentian violet: prepared by mixing 25 cc. of a saturated alcoholic solution of gentian violet with 75 cc. of a solution composed of 5 cc. commercial formalin and 95 cc. distilled water.

Samples of crystal violet with a stated dye content are now available, but were not on hand when this work was started so that the formalin gentian violet solution was made up according to the older procedure of using a "saturated alcoholic solution." This formalin gentian violet staining solution has been used in our laboratory for a number of years and has given satisfactory results, both as a simple stain and in the Gram method. It has the advantage of being stable and keeps well for a considerable period.

Ammonium oxalate crystal violet: since the formalin gentian violet is not in common use, a number of comparative tests were run with an ammonium oxalate crystal violet solution as recommended by Hucker for the Gram stain and as outlined in the latest report of the committee on technic (1927).

Gram staining procedure: formalin gentian violet applied for 3 minutes; wash briefly in water; apply Lugol's iodine solution for 2 minutes; wash briefly in water and decolorize in 95% alcohol for 20 to 30 seconds with gentle agitation; basic fuchsin solution (sat. alc., diluted 1 to 10 with distilled water) was used as the counterstain and applied for 30 seconds to 1 minute.

[1]See Conn (1926), also committee on Bact. Technic (1927).

Hucker's modification of the Gram stain was also tried with a few organisms but gave essentially the same results as the foregoing procedure.

India Ink: a preparation from Coleman and Bell, recommended especially for spirochaetes, was used. At first this was diluted 1 to 7 with distilled water before using, in later tests however it was applied undiluted. Identical results were secured in both cases.

Procedure

After some preliminary experimentation, the following procedure was adopted for testing the killing power of the various staining solutions. Cultures were grown in standard nutrient broth for 24 or 48 hours and then used for the tests. All of the staining was done on small cover slips which had been thoroly cleaned and subsequently kept in 95% alcohol. When desired for use they were lifted from the alcohol with forceps, flamed, and then allowed to stand a moment to cool. A loop of the broth culture was then transferred to the cover slip, gently spread over most of its surface and allowed to dry without heating. During the period of drying the cover slips were placed in sterile petri dishes to prevent air contamination.

In a few instances agar slant cultures were used instead of broth. In this event, a loopful of sterile distilled water was applied to the cover slip and a small amount of growth from the agar slant suspended in it as evenly as possible. It was then handled in the same manner as the other preparations.

A word in regard to the use of broth instead of agar slant cultures may be required here. It is conceivable that, when dried, the resultant thin film on the cover slip would serve to protect, to some extent at least, the cells which are embedded in it, so that somewhat different results might be secured in this case as compared with the use of a suspension of cells in distilled water. The use of broth seemed a somewhat more rigorous test of the killing properties of the stains and so it was employed in our work.

After the film of culture had dried on the cover slips, stains were applied for the length of time desired. This was followed by washing with sterile water and drying without the use of heat. Tests for viability were made as follows: each cover slip to be tested was placed in a sterile Petri dish, a loopful or two of sterile distilled water was placed on each stained film and the film rubbed up in it as far as possible. By this procedure many of the stained bacterial cells were detached from the glass cover slip and suspended in the

drop of water. Then melted nutrient agar[2] was poured directly on the cover slip and allowed to flow over the bottom of the Petri dish. In this way organisms from the stained preparation were scattered thruout the agar in the dish and were not concentrated in one place on the cover slip, as they would have been had the agar been poured directly onto a dry preparation. A few comparative tests showed that this procedure gave the best results. Colonies formed by organisms which survived the staining process were easy to detect and the approximate number, when scattered thruout the plate, could be compared very readily with those from unstained controls prepared in the same manner.

The anaerobic organism used in our work, the Welch bacillus, required somewhat different handling. Cultures were grown in milk and after development of the typical stormy fermentation a loopful was withdrawn, applied to the cover slips, dried and stained as before. It should be remembered that milk cultures which have undergone stormy fermentation contain as a rule no spores, but only the vegetative cells of this organism. After staining, each entire cover slip (broken in 2 or 3 pieces if necessary) was transferred to a milk tube and incubated under anaerobic conditions. The appearance of typical stormy fermentation indicated the presence of viable cells. Unstained controls were made in every instance. Anaerobic conditions were secured by the use of pyrogallic acid and a solution of sodium bicarbonate in carbonated water, as recommended by Rockwell (1924).

Results

The germicidal effect of the various stains is summarized in the accompanying table. Each test was run in quadruplicate. In testing the colon organisms in the Gram stain, for example, four separate cover slips were prepared and each put thru the procedure together with several unstained controls. If all of the four stained preparations were found to be negative the result is indicated by a dash; if one of the four was found to contain viable organisms the result is recorded as one plus (+); if two of the four yielded viable organisms the record is given as ++, and so on to +++ where all yielded positive results. In reading the table it should be kept in mind that where a single plus sign appears, negative results were obtained in the three other cases. Usually in such cases there were only a few colonies on the one plate which gave positive results. In most instances where a 4+ is indicated, abundant growth was

[2]Dextrose agar or other special media may be necessary for certain of the yeasts and yeast-like fungi.

secured on every plate and there was very little if any difference exhibited between the stained preparations and the unstained controls.

TABLE 1. ABILITY OF VARIOUS MICRO-ORGANISMS TO SURVIVE AFTER CERTAIN STAINING PROCEDURES

Name by which culture was designated*	Gram	Gentian violet	Crystal violet, Amm. oxalate	Carbol fuchsin	Loeffler's Methylene blue	Methylene blue	India Ink
		1 min.	1 min.	1 min.	1 min.	1 min.	1 min.
Bacillus megatherium.....	—	++++	++++	+++	++++	++++	++++
Bacillus subtilis.	++++	++++	++++	++++	++++	++++	++++
Clostridium Welchii.....	—	—	—	—		++++	++++
Bacterium prodigiosum.....	—	+	—	++++	++	++++	++++
Bacterium coli...	—	+	—	—	+++	++++	++++
Sarcina aurantiaca.....	—	—	—	—	+	++++	++++
Sarcina lutea....	—	—	+	—	+	—	++++
Staphylococcus albus No. 22..	—	—	—	—	++++	+++	++++
Staphylococcus albus No. 23..	—	—	—	—	++++	++++	++++
Staphylococcus aureus No. 4..	—	+++	—	—	++++	++++	++++
Staphylococcus aureus No. 5..	—	—	—	—	++	++++	++++
Staphylococcus aureus No. 10	—	—	—	—	++		++++
Staphylococcus aureus No. 12	—	+	+	++	++++	++++	++++
Saccharomyces cerevisiae.....	—	—	—	—	—?	+++	++++
Saccharomyces albus.....	—	—	—	—	++++	++++	++++
Torula rosea....	—	—	—	—	++++	+++	++++
Monilia albicans	—	—	—	—	++++	+++	++++
Endomyces albicans.....	—	—	—	—	++++	++++	++++

*Identity of each culture has been confirmed by one of the writers.

Only spores appeared able to withstand Gram staining and even here negative results were secured with *Bacillus megatherium*, altho

microscopic examination showed the presence of spores. In this connection it will be recalled that the Welch bacillus was taken from milk tubes which probably contained only vegetative cells. Gentian violet, crystal violet and carbol fuchsin applied for one minute appeared to exert a high degree of killing effect and the only organisms to survive consistently were the spore-formers. The methylene blue stains gave just the reverse effect, most of the organisms survived consistently and in large numbers. The results with India ink are especially interesting for there appeared to be no killing effect whatsoever. In fact, if the India ink solution is allowed to remain exposed to air contamination, some forms will actually multiply to a certain extent in it. It is used most frequently for relief staining and for the demonstration of spirochaetes and other organisms which do not take the ordinary stains readily. It is obvious that preparations containing pathogenic members of this group will require some additional means of sterilization to obviate the possibility of chance infection.

Attention might also be directed especially to *Monilia albicans* and *Endomyces albicans*. The generic classification of these forms is uncertain and several of our cultures were received under different names, tho very closely related if not identical. This organism is held as the etiologic agent of thrush, an infection of the mucous membranes of the mouth. It survived very well when stained by Loeffler's methylene blue, which is frequently applied to smears made from the the mouth or nose.

REFERENCES

COMMITTEE ON BACT. TECHNIC. 1927. Manual of Methods for Pure Culture of Bacteria, Supplement A, 2nd ed. Pub. by Soc. of Amer. Bact., Geneva, N. Y.

CONN, H. J. 1926. Progress in the Standardization of Stains. Stain Tech., **1**, 49.

JANSEN, A. M. 1914. The disinfectant action of certain bacterial stains. J. Infec. Dis., **14**, 255.

ROCKWELL, G. E. 1924. An improved method for anaerobic cultures. J. Infec. Dis. **35**, 581.

RETTGER, L. F. 1913. Hygiene of the bacteriological laboratory. Am. J. Public Health. **3**, 697.

SCHMIDT, A. 1925. Klin. Wochenschr., **4**, 1731. Werden Bakterien durch die übliche Farbetechnik abgetötet?

THURN, O. 1914. Ueber die Lebensfähigkeit an Objektträgern angetrockneter ungefärbter und gefärbter Bakterien. Centralbl. f Bakt., I Abt., Orig. **74**, 81.

A COMPARATIVE STUDY OF SEVERAL SAMPLES OF NEUTRAL RED AND THEIR EFFECTS ON PARAMOECIUM CAUDATUM

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Abstract.—**Several samples of neutral red have been studied; first, for the determination of their relative color intensities, and second, to determine their toxic effects on *Paramoecium caudatum*.**

It was found that each of these dyes varied in physical characters both in dry form and in solution. The duration of life of groups of Paramoecia varied in each dye solution. These differences are probably due, at least in part, to differences in the chemical composition of each dye. This suggests that the varying results obtained by experimenters, in staining living material when using the same kind of dye under uniform conditions, may probably be attributed to these differences of chemical constitution of the dyes.

In the comparison of the toxic effects of the several samples most uniform results were obtained at concentrations of 1:5,000 and 1:10-000. At a concentration of 1:100,000 neutral red was found toxic but the results obtained were not in agreement with those obtained at lower concentrations.

Some months ago, thru the courtesy of Dr. H. J. Conn, four samples of neutral red were received by Dr. A. B. Dawson for study and the determination of their relative toxicities. I am indebted to Dr Dawson for the opportunity of making these studies.

This study was undertaken with a twofold purpose; first, to determine the relative intensities of these dyes at a certain known concentration, and, second, to determine the relative toxicity of each sample.

Physical Characters

The four samples of neutral red received from Dr. Conn were labelled as follows: NX-1, 3815, CX-1 and I.[1] All were American dyes. The first three dyes were in the form of chlorides and the fourth, marked I, was an iodide. A fifth sample, labeled prewar Grübler and obtained from another source, was also included in the comparative tests.

Each of these dyes differed in physical characteristics. Small portions of each sample were placed on white paper and examined in

[1]Sample 3815 is from the same company as NX-1. We understand it is derived from the latter by a process of purification. Sample I was obtained from Phillips and Cohen (see paper cited on p. 123)—*Editor.*

bright light. The sample NX-1 was reddish-brown in color. Sample 3815 was reddish-brown in color but decidedly darker than the NX-1 sample. The CX-1 sample was extremely dark brown but lacked the reddish cast. The one from Grübler was jet black in color. Each of the samples, NX-1, 3815, CX-1, and Grübler, was of the consistency of a coarse powder. The iodide sample adhered in small, soft clumps. This quality was evident even when an attempt was made to break the clumps with a spatula. Some of these clumps were brown, others were black, and all possessed a metallic luster.

For the comparison of relative color intensities a 1:2500 stock solution in distilled water was made from each of the samples. All glassware, including straight-side pyrex test tubes, pipettes, graduates, and watch glasses, was thoroly cleaned and rinsed with distilled water. Corks used in the mouths of the test tubes were boiled and covered with paraffin.

Five cubic centimeters of each sample solution of neutral red were put into five test tubes. The shades of color of these samples were examined against a white background and matched against each other. At a 1:5000 concentration the order of their intensities was 3815, NX-1, Grübler, Iodide and CX-1. Samples 3815, NX-1 and Iodide were distinctly red. The Grübler dye at this dilution has a very faint straw color and its presence is recognized with difficulty. The sample CX-1 at all concentrations is distinctly straw color. In comparing the intensities at 1:5000 concentration the solution 3815 is the most intense, being distinctly red, while the CX-1 is the least intense and has a pronounced straw color.

An attempt was made to bring all the sample solutions to the same color intensity or shade by diluting the 1:5000 solution. Thus the dyes with the more intense shades of color were diluted to match the dye with the least intense shade. Since the CX-1 had the least intense color an effort was made to dilute all the other samples to match it. It was found upon dilution, however, that none of the other solutions would match the CX-1 solution, since the latter was straw color and not a distinct red. The greater the dilution the more evident was the contrast of the color shades of the other solutions with the CX-1 solution.

As the iodide solution at 1:5000 was the next in the order of intensity, the other sample solutions were matched to it. The following dilutions of the Grübler, NX-1, and 3815,at a concentration of 1:5000, were required to match the iodide:

Stain	*Dilution*
Iodide	1:5000
Grübler	1:5100
NX-1	1:5300
3815	1:5500

Decomposition by Sunlight

Early in the month of October 1926 at the beginning of the investigation, 1:5000 solutions in distilled water were prepared from samples NX-1, 3815 and the Grübler dye. The bottles containing the solutions were placed near a window in direct sunlight. After about three weeks the solutions began to become lighter in color intensity. A thin scum formed on the sides of the bottles and a precipitate formed on the bottoms. Periodic examinations of these decomposing solutions were made. By the middle of March 1927 they had become quite clear. The Grübler solution first assumed a light violet and then a straw color. The NX-1 and 3815 solutions became a very clear straw color. From their appearance one could not tell that these solutions were neutral red, but they still gave the crimson color reaction on the addition of acid.

During the month of June 1927 tests showed decomposition of the solutions to be complete. They failed to give a color reaction on the addition of acid, and when tested with living Paramoecia also failed to stain them vitally. Furthermore these solutions were found to be non-toxic to these organisms, even over a period of forty-eight hours.

For a further study of the effects of sunlight on neutral red, solutions of the NX-1, 3815, and Grübler samples were prepared in 1:2500 concentrations in distilled water and exposed to sunlight on February 16 1927. Observations on September 10, 1927 showed the dyes to be greatly decomposed with a heavy precipitate on the bottom of the containers. Solutions of 1:2500 concentration of the samples CX-1 and I[2] were exposed to sunlight on April 3, 1927. Observations on September 6, 1927 show considerable decomposition of the Iodide sample with a heavy precipitate. The CX-1 solution changed very little in color intensity and shade, but formed a slight precipitate.

According to the changes in color intensities and the formation of precipitates the solutions I and CX-1 do not decompose, under uniform conditions, so fast nor to so great a degree as the solutions

[2]Samples CX-1 and I were received at a later date than samples NX-1, 3815 and Grübler.

3815, NX-1 and Grübler. The CX-1 solution appears more stable than the I.

The solutions used for testing relative color intensities were not taken from these bottles but were prepared especially for that purpose and were kept in a dark place.

Toxic Effects on Paramoecium caudatum

Paramoecium caudatum was used for studying the toxic effects of the neutral red. They were cultured in a hay infusion medium at room temperature. With the aid of a binocular microscope a single organism was pipetted from a mixed culture of protozoa and placed in the culture medium. Within a week, a pure rich culture of Paramoecia of the same clone was obtained.

Solutions of each of the five samples of neutral red used in the toxicity tests were prepared in neutral tap-water and kept in a dark place to avoid decomposition by sunlight. Distilled water was found to be toxic to Paramoecia, while on the other hand pond water was found to cause precipitation of the dyes within twenty-four hours. Tap water proved to be quite favorable, since Paramoecia were found to live indefinitely in it.

In making the determinations, the Paramoecia were concentrated by means of a hand centrifuge and care was taken to avoid injuring the organisms by rapid centrifuging. In each test a drop of the concentrated culture of Paramoecia was pipetted into a watch glass containing 10 cc of the dye. This method of adding one drop of culture to 10 cc. of dye solution was selected in order to avoid any considerable variations in the dilution of the dye. The toxic effects were watched under both the binocular dissecting microscope and the low power of the compound microscope. The experiments were made at room temperature and in weak, diffuse light.

In each test the same pipette was used in transferring the centrifuged Paramoecia into the solutions of neutral red. Controls were run in tap water of the same pH as that in which the samples of neutral red were dissolved. These controls were active and normal hours after each test and continued so until discarded.

Individual Paramoecia vary very widely in the degree of susceptibility to neutral red. In all cases the cilia continued beating almost until death, altho free movements usually disappeared sometime previously to this. The moment at which the cilia of the first organism ceased to beat was taken as the beginning of the lethal stage and the time at which the cilia of all the organisms ceased to beat was taken as the end of the lethal stage. Other evidences of injury are swelling, distortion, and elevation of the pellicle.

Motility was observed under the binocular dissecting microscope. When motility ceased the watch glass was then placed under the compound microscope to observe the beating of the cilia. This method afforded little difficulty since after free movements had

TABLE 1. TOXIC EFFECTS OF THE VARIOUS NEUTRAL RED SAMPLES

Test No.	Dye Sample	Appearance of the 1st elevation of the pellicle	Beginning of the lethal stage	End of the lethal stage
		Solutions of 1:5000 Concentration		
(1)	Grübler	4 min.	2 min.	4 min.
(2)	Grübler	5 min.	2 min.	4 min.
(1)	3815	10 min.	3 min.	11 min.
(2)	3815	9 min.	2 min.	10 min.
(1)	NX-1	1 min.	4 min.	14 min.
(2)	NX-1	3 min.	4 min.	18 min.
(1)	Iodide	8 min.	6 min.	18 min.
(2)	Iodide	5 min.	5 min.	18 min.
(1)	CX-1	10 min.	6 min.	30 min.
(2)	CX-1	11 min.	8 min.	28 min.
		Solutions of 1:10,000 Concentration		
(1)	Grübler	5 min.	5 min.	11 min.
(2)	Grübler	5 min.	5 min.	10 min.
(1)	3815	2 min.	2 min.	19 min.
(2)	3815	2 min.	3 min.	17 min.
(1)	NX-1	3 min.	5 min.	25 min.
(2)	NX-1	8 min.	8 min.	25 min.
(1)	Iodide	10 min.	13 min.	42 min.
(2)	Iodide	10 min.	12 min.	38 min.
(1)	CX-1	10 min.	15 min.	60 min.
(2)	CX-1	10 min.	12 min.	53 min.
		Solutions of 1:100,000 Concentration		
(1)	Grübler	45 min.	40 min.	2 hrs. 11 min.
(2)	Grübler	35 min.	37 min.	2 hrs. 15 min.
(1)	3815	31 min.	24 min.	2 hrs.
(2)	3815	20 min.	21 min.	1 hr. 45 min.
(1)	NX-1	28 min.	23 min.	4 hrs. 54 min.
(2)	NX-1	27 min.	25 min.	4 hrs. 31 min.
(1)	Iodide	25 min.	27 min.	3 hrs. 35 min.
(2)	Iodide	25 min.	26 min.	3 hrs. 28 min.
(1)	CX-1	35 min.	35 min.	5 hrs. 19 min.
(2)	CX-1	30 min.	30 min.	5 hrs. 28 min.

ceased the various individuals could be readily observed for ciliary activity.

The toxic effects at various concentrations are shown in Table 1.

It is evident from the foregoing that no two of the samples are identical in their toxic effects. From six to sixteen trials were made in each concentration of dye. Only the two tests in which the Paramoecia lived the longest period of time are given. The order of the toxicity of the solutions determined by the *end* of the lethal stage is as follows:

Solutions of 1:5000 concentration, Grübler⟩3815⟩NX-1⟩I⟩CX-1
Solutions of 1:10000 concentration, Grübler⟩3815⟩NX-1⟩I⟩CX-1
Solutions of 1:100000 concentration, 3815⟩Grübler⟩NX-1⟩I⟩CX-1

The CX-1 solution which has the distinct straw color is the least toxic in all concentrations as indicated above.

The order of the toxicity of the dye solutions determined by the *beginning* of the lethal stage is as follows:

Solutions of 1:5000 concentration, Grübler⟩3815⟩NX-1⟩I⟩CX-1
Solutions of 1:10,000 concentration, 3815⟩Grübler⟩NX-1⟩I⟩CX-1
Solutions of 1:100,000 concentration, 3815⟩NX-1⟩I⟩CX-1⟩Grübler

In general the reactions of Paramoecia to neutral red are identical to those when the organisms are treated with mineral acid but there are some minor variations.

These reactions have been pointed out by Jacobs (1920) and are as follows: "A mineral acid, such as HCl or H_2SO_4 clearly acts from without inward, killing or injuring its way as it goes. One of the first effects produced by it is on the cilia, locomotion ceasing and their beat becoming slow and irregular. At a time when the beat of the cilia has almost ceased, the internal structures in the cell may be apparently unaffected. The contractile vacuoles may pulsate regularly, the protoplasm be clear and transparent and the nuclei possess their normal appearance. As the acid slowly enters the cell, the pulsations of the contractile vacuoles cease, swelling of the body occurs, the nuclei stand out sharply from the cytoplasm and the latter becomes coagulated and opaque."

We may add that in each sample solution of 1:5000 concentration, trychocysts are discharged and later a clear pellicle appears either near the mouth or at the anterior end. If locomotion persists, it is quite spasmodic and irregular. Few may dart here and there until they succumb to the toxic effects of the dye. In some instances the body swells, the cell wall breaks and the protoplasm flows out. In most cases those that die most quickly after treatment show no lifting of the pellicle. However, their bodies may become greatly distorted. This is indicated in the tabulated results of solutions of 1:5000 concentration where the time of the appearance of the first pellicle is longer than the beginning of the lethal stage.

In the 1:10,000 and 1:100,000 concentrations either the pellicle is evident before any of the group dies, or the pellicle appears simultaneously with death.

In solutions of 1:10,000 concentrations, trychocysts are discharged very soon after treatment, the organisms are stimulated, locomotion is rapid and usually jerky. However, the organisms begin to slow down after a few minutes.

In solutions of 1:100,000 the dye enters almost as rapidly as in solutions of higher concentrations. It is quite apparent within 30 seconds after treatment but varies in intensity in different individuals, demonstrating differences in the reaction of protoplasm to the same agent under apparently identical conditions. These differences in color shades persist for two or three minutes after treatment. However very soon after they all become about the same shade.

It has been pointed out elsewhere by Tröndle (1920) that concentration has little "effect on the rate of absorption." According to Child and Deviney (1926) neutral red and methylene blue penetrate the membrane of Paramoecia almost immediately "even in darkness."

When first treated with solutions of 1:100,000 concentration locomotion is generally normal from five to ten minutes before slowing down or exhibiting any irregularity.

Discussion

The varying results obtained by different experimenters in vital staining when using the same method and the same stains or dyes, may be explained by the assumption that altho identical in name the dyes may vary considerably in chemical composition. No two of the five samples of neutral red in this investigation are identical. These samples vary in physical characters, in color intensities at the same concentration, and in their physiological effects on Paramoecium under the same conditions. Thus we may assume that they vary in chemical composition. Several methods for making the neutral red iodide have been pointed out by Phillips and Cohen (1927) who state further that satisfactory stains were attained only when the preparation was free from toluylene blue. The necessity of an investigator knowing the "chemical constitution, quantitative as well as qualitative, of a stain which is being used in the study of living material" has been pointed out and emphasized by Dawson (1926 a-b)

On the other hand, some investigators have looked elsewhere for causes that vary the reactions of protoplasm when the same agents are used under identical conditions. Certainly the variations are due either to: (1) conditions inherent in the structure of protoplasm;

(2) conditions in the environment; or (3) the agent itself as pointed out in this investigation. Lyons (1902, 1904) Spalding (1904) Matthews (1906) and R. S. Lillie (1916) have shown eggs to be more susceptible to certain agents just before or during the time of cell division. Whether this principle can be applied to Paramoecium we can not state. It is apparent that age influences the susceptibility of Planaria, for the young and starved "show a greater degree of susceptibility than the older worms and well fed worms, respectively," (Child, 1915).

On the other hand as indicated by B. L. Lund (1918) "young Paramoecia and starved Paramoecia are more susceptible to KCN than old (fed) Paramoecia." Petersen (1927) working on *Paramoecium caudatum* "found that in the isolated fission products of animals which had divided in the morning the anterior piece had a more rapid rate, but in those isolated in the evening after division, the posterior piece divided first." He also found that "there may be an inheritance of the relative regional metabolic rates of the original animal in the fission products at least to the third filial generation." It was found that "feeding a starving Paramoecium with yeast or yolk of hen's egg increases the rate of CO_2 production from two to three times," (E. J. Lund, 1918). The physiological conditions differing in the tail region of different Paramoecia has also been pointed out by Child and Deviney (1926). The apical end of Grantia and Tubularia were found by Hyman (1924) "to respire at a more rapid rate than the basal piece."

There is no doubt that age, temperature, light, food, fission products of anterior and posterior end of Paramoecia and metabolic rates may influence the susceptibility of individua organisms under apparently the same conditions. Yet we can not overlook the fact that differences in the kind of dye may exist which may account for the varying results obtained in staining living tissue, especially if these relative differences are constant and consistent with groups of organisms under apparently identical conditions.

The extreme variability in susceptibility of *Paramoecium caudatum*, especially in the highest dilution (1:100,000) of these dyes, would appear to indicate that this dilution is not well adapted for studies of relative toxicity. As concentrations of 1:5000 and 1:10,000 the results are quite uniform whether the beginning or end of the so-called lethal stage is taken as a criterion for comparing toxic effects.

LITERATURE CITED

CHILD, C. M. 1915. Senescence and rejuvenescence. Chicago.

CHILD, C. M. and DEVINEY EZADA. 1926. Contributions to the physiology of *Paramaecium caudatum*. J. Exp. Zool. **43,** 257.

DAWSON, ALDEN B. 1926a. The labelling of biological stains. Stain Techn. **1,** 96.

1926b. The adjustment of concentration of vital stains by colorimetric methods. Stain Techn. **2,** 19.

HYMAN, L. H. 1924. Respiratory differences along the axis in Grantia and Tubularia. Proc. Amer Soc. Zoologists Anat. Rec., **29,** 91.

JACOBS, M. H. 1920. To what extent are the physiological effects of carbon dioxide due to hydrogen ions? Amer. J. Physiol., **51,** 321.

LILLIE, R. S. 1916. Rhythmical changes in the resistances of the dividing sea urchin egg to hypertonic sea water and their physiological significance. J. Exp. Zool. **21,** 369.

LUND, BARBARA L. 1918. The toxic action of KCN and its relation to the state of nutrition and age of the cell as shown by Paramoecium and Didinium. Biol. Bull., **35,** 211.

LUND, E. J. 1918. Quantitative studies on intracellular respiration. II. The rate of oxidation in *Paramoecium caudatum* and its independence of the toxic action of KCN. Amer. J. Physiol. **45,** 365.

LYONS, E. P. 1902. Effects of KCN and of lack of O_2 upon fertilized eggs and embryos of the sea urchin. Amer. J. Physiol. **7,** 56.

1904. Rhythms of susceptibility and of CO_2 production in cleavage. Amer. J. Physiol. **11,** 52.

MATTHEWS, A. P. 1906. A note on the susceptibility of segmenting Arbacia and Asterias eggs to cyanides. Biol. Bull. **11,** 137.

PETERSEN, W. 1927. The axial gradient in Paramoecium. Science **66,** 157.

PHILLIPS, M. and COHEN, B. 1927. The preparation of vital neutral red. Stain Techn. **2,** 74.

SPAULDING, E. G. 1904. The rhythm of immunity and susceptibility of fertilized sea urchin eggs to ether, HCl, and to some salts. Biol. Bull, **6,** 224.

TRÖNDLE, A. 1920. Neue Untersuchungen über die Aufnahme von Stöffen in die Zelle. Biochem. Ztschr. **112,** 259. Cited from E. V. Cowdry, General Cytology.

EXTINCTION COEFFICIENTS OF DYES

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Extinction coefficients (k), or the specific transmissive indices[1], as determined spectrophotometrically, are necessary in a quantitative study of dyestuffs with a spectrophotometer. Such data are distinctive for individual dyes, and may be employed in two ways: with stains of *unknown* character, but whose dye content can be determined by some other method, the extinction coefficient serves to establish the nature of the dye; or with stains of *known* identity, the extinction coefficient is a criterion of dye content.

These values are constant for a given dye in a given concentration and solvent when examined in a layer of a certain thickness. By definition the concentration of 10 parts of dye in a million of solvent with a cell depth of 1.0 cm. is accepted as standard.

Variations in concentration in some solvents result in a marked change in the spectrophotometric absorption curve independent of chemical variations of the dyestuff.[2] In view of this, especially in the case of basic dyes, 50% alcohol is used as a solvent. While in these determinations water was the solvent in the case of acid dyestuffs, 50% alcohol was used with basic dyes and will be employed in this laboratory in all subsequent work irrespective of dye.

Due to the uncertainty of composition in the case of many samples of dye, results are approximate, but as additional samples are determined the data will become of a progressively higher degree of accuracy. The titanous chloride titration and other chemical determinations of dye content upon which this work is based are subject to considerable inaccuracy which effect spectrophotometric values also.

In the following table the acid and basic dyes have been grouped as values of k vary appreciably with different solvents; k has been determined for two or more wave lengths in the vicinity of maximum absorption.

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[1]Gibson, K. S. et al. The spectral transmissive properties of dyes: I. Seven permitted food dyes, in the visible, ultra-violet, and near infra-red. U. S. Bur. Standards, Sci. Papers, No. 440. 1922.

[2]Holmes, W. C. The influence of variation in concentration on the absorption spectra of dye solutions. Ind. & Eng. Chem. **16**, 35–40, 1924. Washington.

French, R. W. Effect of variations in concentration of dyes in solution upon their quantitative determination spectrophotometrically. Methylene blue. Ind. & Eng. Chem. **18**, 298–9. 1926.

EXTINCTION COEFFICIENTS (k)

ACID DYES	mμ	k	No. of Samples
Eosin B	510	0.770	1
	520	0.876	
Rose bengal	540	0.778	1
	550	0.924	
Congo Red (alkaline form)	500	0.578	1
	510	0.558	
(acid form)	660	0.475	1
	670	0.479	
Orange G	480	0.552	2
	500	0.527	
Phloxine	530	0.851	1
	540	1.060	
Eosin Y	516	1.315	6
	520	1.234	
Acid fuschsin	530	0.989	2
(sulphonated new fuchsin)	540	1.206	
Acid fuchsin	530	0.885	2
(sulphonated *p*-rosanilin)	540	1.038	
Acid fuchsin	530	1.117	6
(sulphonated rosanilin)	540	1.385	
BASIC DYES			
Methyl violet 2B	570	2.002	4
	580	2.226	
Safranin	535	1.531	4
	545	1.292	
Crystal violet	580	2.440	3
	590	2.777	
Para-rosanilin (chloride)	540	1.609	1
	545	1.733	
	550	1.644	
Rosanilin (chloride)	540	2.283	1
	545	2.524	
	550	2.577	
New fuchsin (chloride)	540	2.199	1
	545	2.524	
	550	2.585	
Brilliant cresyl blue	620	1.346	1
	630	1.383	
Methyl green	630	0.396	1
	640	0.514	
Cresyl violet	620	0.678	1
	630	0.715	

THE HISTORY OF STAINING

A PRELIMINARY NOTE

H. J. Conn

When this journal was christened Stain Technology, the name seemed at first a little presumptuous, as tho implying that the subject treated were a science in itself. Stains have always been considered merely as tools of the biological sciences, and the thought that there might be a science involved in the study of their nature and uses was a new idea which seemed likely to provoke a smile rather than to call for serious consideration. Yet without regarding Stain Technology as a science, it must now be granted that the study of stains and their uses has developed so many unexpected aspects of interest and importance to biology that it does prove worthy of much time and attention on the part of scientists. The significance attached to it it at present seems to be one of the outgrowths of the cooperative spirit that has developed among scientists in recent years. One or two instances that stand out in the writer's mind illustrate the growth of the idea.

In the fall of 1920 a group of men, presided over by Dr. L. R. Jones, in his capacity as chairman that year of the Division of Biology and Agriculture of the National Research Council, gathered together in New York City for the sole purpose of discussing biological stains. At this meeting were other well known scientists, such as Dr. R. A. Harper, Dr.H. E. Howe, and Dr. C.E. McClung; while the practical side was represented by Mr. R. T. Will of Rochester, who had first foreseen the value of cooperative work on stains, and Mr. W. F. Keohan of the Chemical Foundation, whose interest in this subject, begun at this time, has been growing ever since. A meeting to discuss biological stains, representing such varied interests and talents, had certainly never been held before. Perhaps every one present was somewhat surprised to find such a subject considered important enough to be receiving so much time from prominent scientists and busy men of affairs.

Altho this meeting had for its object the cooperative study of stains with the idea of securing reliable products in America, no one present dreamed that it was opening up a new and very instructive line of research. The use of the term Stain Technology by anyone at that meeting would have been inconceivable; and if some one had suggested a journal to cover this field, the idea would possibly have been greeted with a polite but incredulous smile.

The second incident occurred nearly five years later. It was a conference between Mr. F. P. Garvan, president of the Chemical Foundation, and the writer, held in the former's office in New York. After promising the assistance of the Chemical Foundation on a greater scale in the future than in the past, Mr. Garvan suddenly asked: "Is there any scientific journal covering this subject?" upon receiving reply in the negative, he immediately went on: "Well, there will be some day! Why not pre-empt the field?"

This was the first occasion that the study of stains and their action, as an integral branch of science, had been suggested to the writer's mind. It seemed also extremely significant that the idea had first occurred to someone who was not a scientist, altho well known to be inspired by a realization of the value of science to the future of his country.

The idea of Stain Technology, first put in words on this occasion, not only gave origin to the journal, but also has had much to do with the direction taken by the research carried on and organized by the stain commission. The idea which is gradually shaping itself is that Stain Technology, altho not an end in itself, is a branch of investigation which has for its object the improvement of certain tools used in the biological sciences.

Regarding this subject as an integral branch of investigation, it becomes of interest to trace out its history. It is well known that dyes were first used in microscopic work about the middle of the last century and that since then the lines of their application have been increased from year to year. The history of the subject is touched briefly in Biological Stains; but it has been suggested that a more detailed account of the history of staining would be of value to teachers and of interest to investigators in these fields. Accordingly an effort is being made to get together the facts concerning the matter; and it is planned to publish them in a series of papers in Stain Technology. The preparation of these papers will be a coöperative undertaking; and it is hoped that when they are completed they will prove valuable enough to be reprinted in book form. The first of these papers, dealing with early staining methods, will probably appear in the January number.

ERRATA IN VOL. 2

P. 69. Transpose first and second lines.
P. 72, line 13. After words "buffer solution" add: (pH-value 7.35)

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